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Application of an electrochemical detector with a graphite electrode to liquid chromatographic determination of penicillamine and captopril in biological samples

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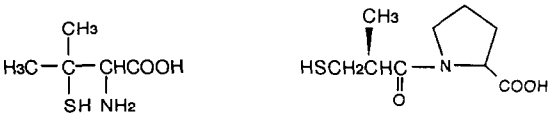
Abstract: A high-performance liquid chromatographic determination of penicillamine and captopril in rat serum, liver and kidney samples is described. An electrochemical detector with a graphite working electrode at a potential of +0.9 V vs the Ag/AgCl reference electrode is used for the detection system. Linear responses of the peak height to the amount of samples injected were obtained in a range of 0.1–500 ng on-column and 0.5–500 ng on-column for penicillamine and captopril with correlation coefficients of 0.997 and 0.995, respectively. Detection limits at a signal-to-noise ratio of 3 were 20 and 300 pg for penicillamine and captopril, respectively. The graphite electrode has a long lifetime of about 4 months with continuous use, even with the high voltage supplied. The analytical application of this method to the determination of penicillamine and captopril in biological samples was successful.

Keywords: *Penicillamine; captopril; serum and tissues; electrochemical detection; reversed-phase HPLC; graphite electrode.*

Introduction

D-Penicillamine (α-amino-β-methyl-β-mercaptobutanoic acid) and captopril (1-(D-3-mercapto-2-methyl-1-oxopropyl)-L-proline) are sulphhydryl-containing pharmaceuticals (Fig. 1). Penicillamine is an important drug in the therapy of rheumatoid arthritis [1], Wilson's disease [2] and in the treatment of metal intoxication [3]. Recently, it has also been reported that penicillamine selectively inhibits the replication of human acquired immunodeficiency syndrome (AIDS) virus [4]. Captopril is a potent and specific active antihypertensive drug, designed to inhibit angiotensin converting enzyme *in vivo* [5, 6]. In order to study the pharmacokinetics of penicillamine and captopril in human and animal, a rapid, sensitive and selective assay for these drugs in biological samples is necessary. Several high-performance liquid chromatographic (HPLC) methods are reported for the determination of penicillamine and/or captopril in biological samples based on spectrophotometry [7–9] or fluorometry [10, 11]. The recent development of electrochemical detection (ED), including a glassy carbon electrode [12, 13] and mercury-based electrochemical detector [14–23] has provided a sensitive assay for thiols. The latter detection shows a marked specificity for the assay of thiol compounds, however, it was subjected to electrode passivation phenomena. Average lifetimes of mercury-based electrode are reported to be from 2 to 3 weeks at the longest [20, 23]. That is to say, the mercury-based electrode is difficult in terms of keeping the sensitivity constant, therefore, renewal of the electrode is frequently required using polishing



D-penicillamine **captopril**

Figure 1
Chemical structures of D-penicillamine and captopril.

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and resurfacing [23]. Glassy carbon electrodes can fully exhibit electrochemical responses and stabilities under non-aqueous solvent conditions. In aqueous mobile phase, glassy carbon electrodes tend to increase non-specific adsorption of substances on the active surface, and then diminish electrochemical responsiveness. On the other hand, graphite electrodes have superiority in some respects to glassy carbon electrodes, e.g. economical, easy to handle and exhibit very low residual current and noise [24].

In this paper, a graphite electrode has been used instead of a mercury-based or glassy carbon electrode for the electrochemical determination of penicillamine and captopril, and applied to the assay of drug levels in rat serum, liver and kidney.

Experimental

Reagents

D-Penicillamine and captopril were purchased from Sigma (St Louis, MO, USA). Sodium octyl sulphate was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals and solvents employed were of analytical reagent or HPLC grade, and were used without further purification.

Apparatus and chromatographic conditions

A Shimadzu model LC-6A high performance liquid chromatograph (Shimadzu, Kyoto, Japan), equipped with a Eicom model ECD-100 electrochemical detector (Eicom, Kyoto, Japan) and a Shimadzu Chromatopac C-R3A integrator (Kyoto, Japan) was used. The applied potential of the graphite working electrode WE-3G (Eicom) was set at +0.9 V vs an Ag/AgCl reference electrode. The samples were introduced by means of a Rheodyne model 7125 sample injector (100-μl loop, Cotati, CA, USA). A Shodex C_{18} column (150 × 4.6 mm i.d., Showa Denko, Tokyo, Japan) was used under ambient conditions. The following mobile phases were used at a flow-rate of 0.8 ml min^{-1}: (1) 0.05 M phosphate buffer (pH 2.8)–methanol (95:5, v/v) for penicillamine analysis; (2) 0.05 M phosphate buffer (pH 5.5)–methanol (95:5, v/v) for captopril analysis. Each mobile phase contained sodium octyl sulphate (1 mM) and EDTA (10 μM). Prior to use, the mobile phase was filtered through a 0.4-μm membrane filter and degassed.

Sample preparation of rat serum, liver and kidney

D-Penicillamine and captopril were individually dissolved in 0.9% saline, and the concentrations were 150 mg ml^{-1} and 25 mg ml^{-1} for penicillamine and captopril, respectively. Male Wistar rats, weighing 250–300 g were used for experiments. Solutions were orally administered at a dose of 300 and 50 mg kg^{-1} for penicillamine and captopril, respectively. After 1 h administration, the rats were killed by decapitation, and liver and kidney tissues were quickly removed and rinsed in ice cold saline, and stored at −80°C prior to analysis. Blood was collected by venipuncture into centrifuge tubes, and centrifuged (1500*g*, 10 min) at room temperature. The sera thus obtained were stored at − 80°C until assayed.

Assay of penicillamine and captopril in rat

Serum sample. To 0.1 ml of serum, 50 μl of 0.1% EDTA solution and 0.2 ml of 10% trichloroacetic acid (TCA) solution were added. For penicillamine assay, 50 μl of homocysteine solution (32 μg ml^{-1}) as an internal standard was then added to the mixture. The mixture was vortex-mixed for 30 s, and was centrifuged (1500*g*, 10 min). The supernatant was filtered through a Column Guard (Millipore). Five-microlitre aliquot for penicillamine or 50 μl aliquot for captopril was injected into the HPLC-ED system, respectively.

Liver and kidney samples. Liver and kidney tissues (*ca* 0.1 g of wet weight) were minced and homogenized in 0.2 ml of 0.1% EDTA solution and 0.2 ml of 10% TCA solution, and then centrifuged (1500*g*, 10 min). For penicillamine assay, 50 μl of homocysteine solution (64 μg ml^{-1}) as an internal standard was added to the mixture before homogenization. The supernatant was loaded to a Bond Elut C_{18} solid-phase extraction cartridge (Varian, Harbor City, CA, USA) previously conditioned with successive 2 ml of methanol, water, and phosphate buffer (pH 3.0; 0.01 M). After loading, the cartridge was pre-treated with successive 0.5 ml of phosphate buffer (pH 3.0; 0.01 M) and 5% methanol. The desired fraction was then eluted with 0.5 ml of methanol. The eluent was evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in mobile phase (200 μl), and 5 μl aliquot for penicillamine assay or 50 μl

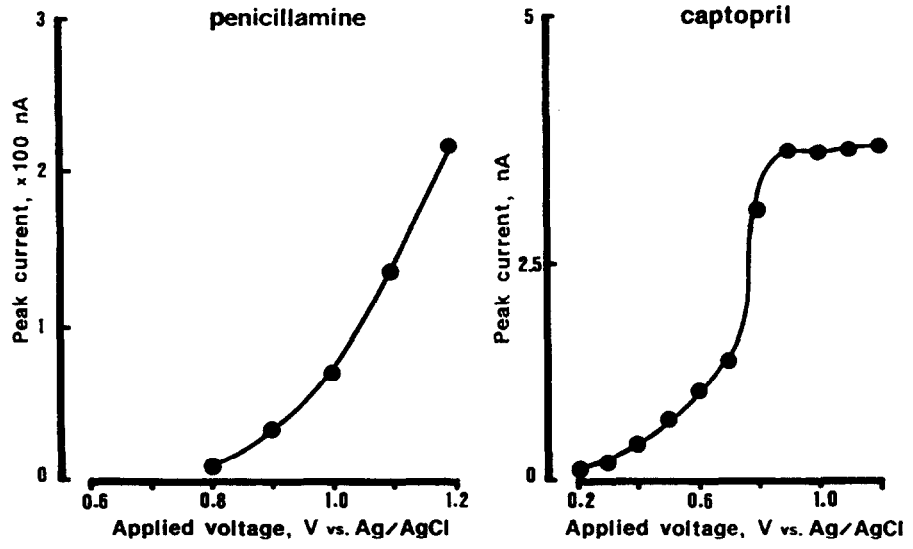

Figure 2
Hydrodynamic voltammograms of penicillamine and captopril on graphite electrode.

aliquot for captopril assay was applied to the HPLC-ED.

Results and Discussion

The graphite electrode has a hydrophilic surface and so should be adequate under aqueous mobile phase conditions. Penicillamine and captopril were readily detected at the graphite electrode at a potential of +0.8 and +0.2 V, respectively (Fig. 2). When the potential of the graphite working electrode was set at +0.9 V vs the Ag/AgCl reference electrode, the background current was constantly less than 10 nA. Shaw *et al.* [12] employed a glassy carbon electrode for HPLC analysis of penicillamine in plasma, so we have also investigated the use of glassy carbon electrode (WE-GC, Eicom) for comparison. The detector response of the glassy carbon electrode for the thiol compounds was 40% less than that of the graphite electrode under the experimental conditions employed, and the background current of glassy carbon electrode was more than 50 nA. The difference of the background current might arise from the characteristics of the electrode to mobile phase polarity. To achieve the optimum resolution, several kinds of column (Capcell Pak C_{18}, Shodex C_{18}, Inertsil C_{18}, Zorbax ODS and Shodex RS pak) were examined using six different thiol compounds, i.e. cysteine, *N*-acetylcysteine, glutathione, homocysteine, penicillamine and captopril. Considerable differences among individual columns were noted and Shodex C_{18} (150 × 4.6 mm i.d.) provided the best separation of thiols. The chromatographic behaviour of penicillamine and other thiol compounds have been well described by Allison *et al.* [19]. On the basis of their results, methanol concentrations, concentrations of sodium octyl sulphate as an ion-pairing agent in the mobile phase, the pH values of eluting buffer, and flow-rates were examined for optimizing the elution conditions. Using 5% methanol with sodium octyl sulphate (1 mM), pH value of 2.8, and a flow-rate of 0.8 ml min^{-1}, thiol compounds were successfully resolved from each other without interference. Figure 3(a) shows a typical chromatogram of penicillamine and other thiol compounds obtained under these conditions. The retention time and the detection limit of penicillamine were 8.3 min and 20 pg (a signal-to-noise ratio of 3), respectively. In the case of captopril elution, it required about 30 min under this condition, so the pH value of the eluting buffer was adjusted to 5.5. The elution of captopril was accelerated, and the retention time was shortened to 8.9 min (Fig. 3b). Detection limit of captopril was 300 pg (a signal-to-noise ratio of 3). The detection limits of penicillamine and captopril with the present method were lower than those with HPLC method reported earlier [12, 16, 17]. Retention times, peak areas, and peak heights of penicillamine and captopril were extremely reproducible. The relative standard deviations of

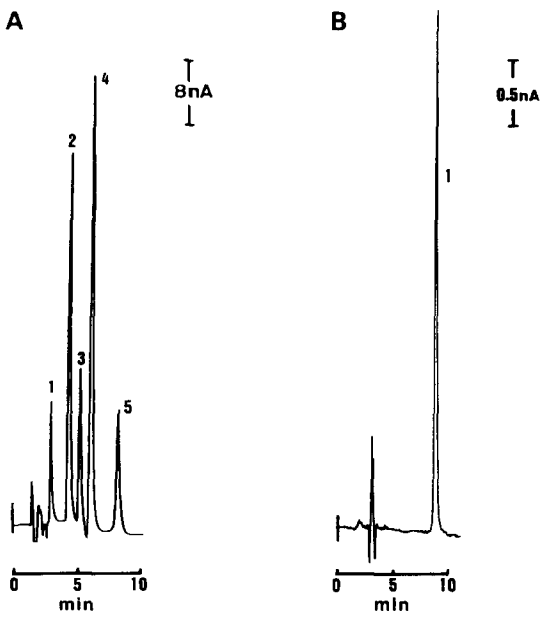

Figure 3
Chromatograms of authentic thiol compounds. Chromatogram (a): peak 1 = cysteine, 2 = *N*-acetylcysteine, 3 = glutathione (reduced form), 4 = homocysteine, 5 = penicillamine. The amount of individual compounds was 40 ng per 5 μl injection. Chromatogram (b): peak 1 = captopril. The amount of captopril was 20 ng per 50 μl injection.

peak area and peak height for each compound were less than ± 6%. For penicillamine over the range of 0.1–500 ng on-column, using seven standard solutions assayed five times each, the regression equation was $y = 0.017x + 0.045$, correlation coefficient (r) = 0.997 (n = 35). For captopril over the range of 0.5–500 ng on-column, the regression equation was $y = 16.4x + 28.9$, $r = 0.995$ (n = 35). Peak height ratios were used for penicillamine and peak heights for captopril. The deproteinizing agents, trichloroacetic acid (TCA), perchloric acid, methanol, ethanol, and acetonitrile were compared for their suitability. TCA solution produced the cleanest baseline and was considered most suitable. The clear filtrate obtained from deproteinized serum can be introduced to the column without further pretreatment. The chromatograms from liver and kidney homogenates samples contained a broad unknown peak at the retention time of 40 min. A Bond Elut C_{18} solid-phase extraction cartridge was found to be effective to remove the unknown substances. Penicillamine and captopril were eluted from the column with 0.5 ml of methanol, but the unknown substances were retained in the cartridge. The chromatograms of the samples from rat serum, liver and kidney show no interfering peaks for penicillamine and captopril assays. The recovery results of penicillamine and captopril spiked to rat serum, liver, and kidney at two different concentrations are shown in Tables 1 and 2, and are considered satisfactory. The proposed HPLC-ED method was applied to the determination

Table 1
Analytical recovery of penicillamine added to rat serum, liver and kidney

	Penicillamine added (μg)	Recovery (%) mean ± SD*	(RSD %)
Serum (100 μl)	0.1	96.4 ± 4.5	4.7
	1.0	98.2 ± 3.5	3.6
Liver (0.1 g)	0.1	94.7 ± 6.1	6.4
	1.0	96.1 ± 4.9	5.1
Kidney (0.1 g)	0.1	93.2 ± 4.7	5.0
	1.0	96.3 ± 4.2	4.4

*n = 5.

Table 2
Analytical recovery of captopril added to rat serum, liver and kidney

	Captopril added (ng)	Recovery (%) mean ± SD*	(RSD %)
Serum (100 μl)	10	95.7 ± 5.5	5.8
	100	97.6 ± 3.9	4.0
Liver (0.1 g)	10	90.9 ± 6.7	7.4
	100	92.5 ± 5.4	5.8
Kidney (0.1 g)	10	92.4 ± 6.3	6.8
	100	93.1 ± 5.2	5.6

*n = 5.

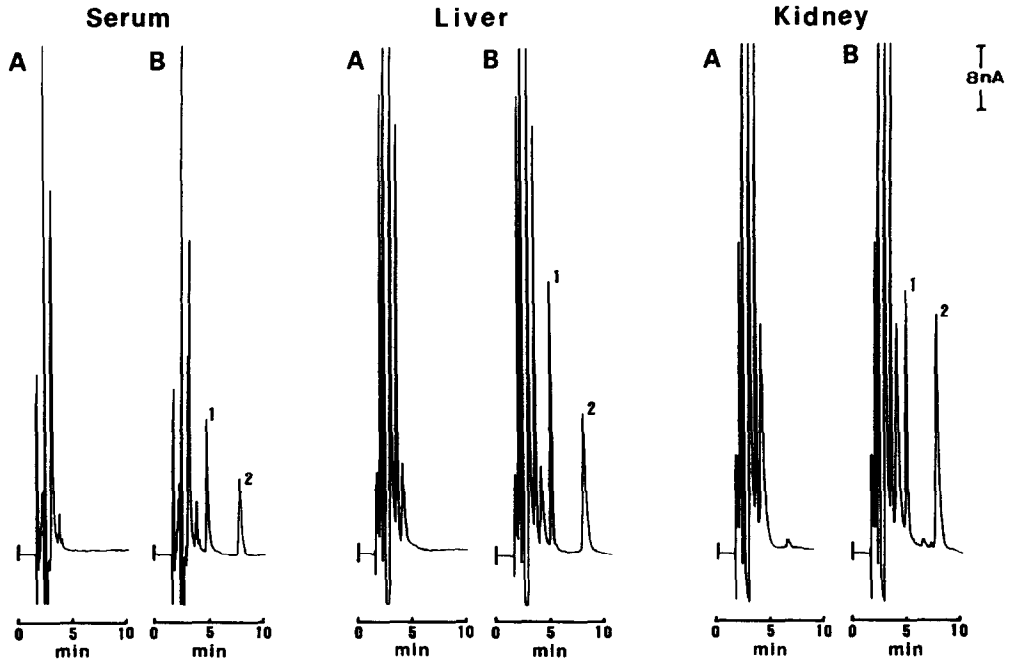

Figure 4
Typical chromatograms of (a) drug-free rat sample and (b) sample taken from a rat 1 h after oral treatment with 300 mg kg^{-1} penicillamine. Peaks: 1 = homocysteine (internal standard); 2 = penicillamine. The injection volume was 5 μl. The details are in the text.

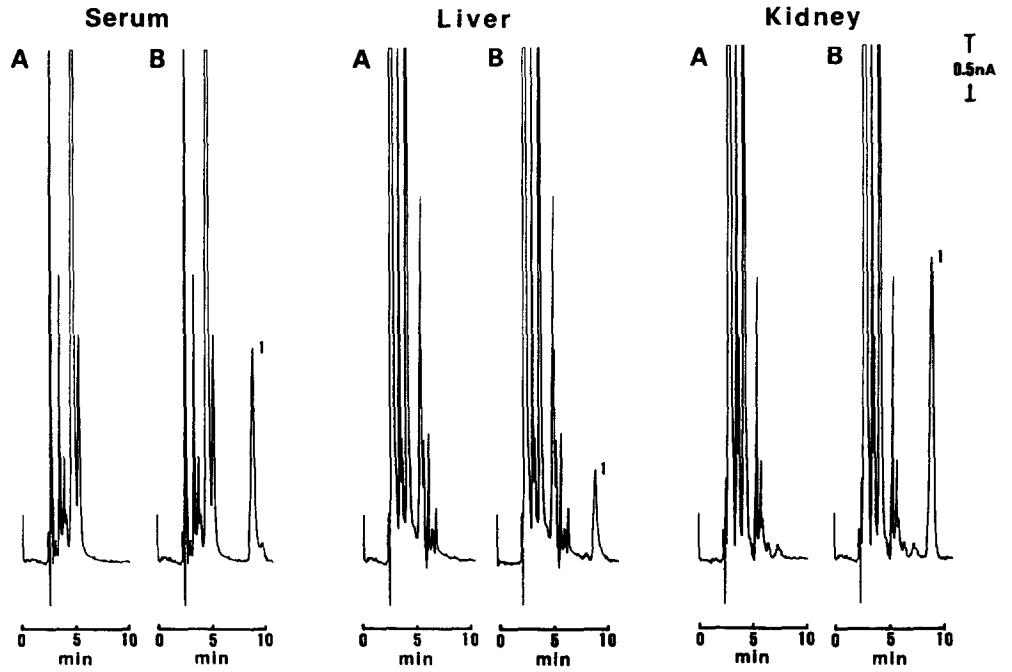

Figure 5
Typical chromatograms of (a) drug-free rat sample and (b) sample taken from a rat 1 h after oral treatment with 50 mg kg^{-1} captopril. Peak: 1 = captopril. The injection volume was 50 μl. The details are in the text.

of penicillamine and captopril in rat serum, liver, and kidney samples. The typical chromatograms of serum and tissues are shown in Figs 4 and 5. Figure 4 shows the chromatograms of (a) drug free samples and (b) samples taken from a penicillamine-administered rat. Figure 5 shows the chromatograms of (a) drug free samples and (b) samples taken from a

Table 3
Concentration of penicillamine 1 h after a 300 mg kg^{-1} oral dose in rat serum, liver and kidney

	Mean ± SD*
Serum	37.2 ± 5.8 μg ml^{-1}
Liver	64.5 ± 10.1 μg g $tissue^{-1}$
Kidney	112.8 ± 17.7 μg g $tissue^{-1}$

*n = 5.

Table 4
Concentration of captopril 1 h after a 50 mg kg^{-1} oral dose in rat serum, liver and kidney

	Mean ± SD*
Serum	0.58 ± 0.11 μg ml^{-1}
Liver	0.30 ± 0.05 μg g $tissue^{-1}$
Kidney	1.36 ± 0.34 μg g $tissue^{-1}$

*n = 5.

captopril-administered rat. The distributed concentrations of drugs in rat serum, liver, and kidney were measured after 1 h of a single administration at a dose of 300 mg kg^{-1} for penicillamine and 50 mg kg^{-1} for captopril, respectively. Drug levels are summarized in Tables 3 and 4. The method described here is sensitive enough to determine the drug levels in serum and tissues, using a small amount of serum (0.1 ml) or tissues (0.1 g). There are many reports for the determination of penicillamine or captopril in blood and/or urine, but are few data about the distribution of penicillamine and captopril in tissues. The drug levels in liver and kidney are in accordance with those obtained by the radiometric method [25, 26].

The stable responsiveness of the graphite electrode was maintained for about 4 months with continuous use, even though a high voltage was supplied for the assay of biological samples. The decrease of electrode sensitivity was recovered by wiping the surface of electrode with acetone. Graphite electrode WE-3G (Eicom) is superior to other electrodes with regard to reproducibility and accuracy under the aqueous mobile phase employed, although the specificity of the graphite electrode for thiols is inferior to that of a mercury-based electrode. A glassy carbon electrode should be chosen when mobile phase contains more than 30% v/v methanol. The method is suitable for use in pharmacokinetic and pharmacodynamic investigations of penicillamine and captopril.

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